ANALYTICAL BIOCHEMISTRY 86, 692–696 (1978)

A Method for Separation of Trehalose from Insect Hemolymph

KARL J. KRAMER, ROY D. SPEIRS, AND CYNTHIA N. CHILDS[1]

U. S. Grain Marketing Research Laboratory, Agricultural Research Service, U. S. Department of Agriculture, Manhattan, Kansas

Received August 8, 1977; accepted January 3, 1978

A method was developed for the determination of trehalose levels in insect hemolymph. The disaccharide is first purified by gel-permeation chromatography and then quantitated by the anthrone colorimetric procedure. The concentration of trehalose in hemolymph from eleven species is reported. The method is applicable to determinations in other tissues and organisms as well.

In many insect species, trehalose (α-D-glucopyranosyl-α-D-glucopyranoside) is the predominant circulating saccharide that plays a central role in carbohydrate metabolism (18). In order to understand the physiological interactions of this metabolite with regulatory agents, precise determinations of trehalose levels in tissues are essential. The presence of other saccharides makes a direct *in situ* analysis virtually impossible and has led several laboratories to use indirect methods to measure trehalose. A common procedure involves the use of trehalase to convert the disaccharide to glucose, which is then measured by the specific glucose oxidase enzymatic assay (5–7). The difficulty here is that trehalase and glucose oxidase regulate trehalose, glucose, and glycogen levels *in vivo* (4) and are subject to inhibition by other substances found in blood or tissue extracts such as hormones, ions, and metabolites (10, 12–14). Another method requires the use of a gas–liquid chromatograph after the sample has been deproteinized, extracted, and silylated (2). The present study was undertaken to develop a simple, direct, and quantitative spectrophotometric method for trehalose determination that would not be subject to interference by other components in tissues and that could be set up rather inexpensively. Our procedure consists of purifying trehalose by gel-permeation chromatography, followed by direct quantitation using the anthrone reagent.

[1] Research Chemist, Research Entomologist, and Research Assistant, respectively.

0003-2697/78/0862-0692$02.00/0

MATERIALS AND METHODS

Insects. The tomato hornworm, *Manduca quinquemaculata* (Haworth), and chersis *moth larva, Sphinx chersis* (Hubner), were collected from tomato plants and ash trees, respectively. The tobacco hornworm, *Manduca sexta* (L.), and American cockroach, *Periplaneta americana* (L.), were obtained from laboratory colonies reared on the agar-based diet of Bell and Joachim (3) and on dog food, respectively. Hemolymph of the milkweed bug, *Oncopeltus fasciatus* (Dallas), was a gift of Dr. Sukh Bassi, Benedictine College, Achison, Kansas. These animals were cultured on milkweed seeds. Other insects [Indian meal moth larva, *Plodia interpunctella* (Hubner), navel orange worm, *Paramyelois transitella* (Walker), almond moth larva, *Cadra cautella* (Walker), Mediterranean flour moth larva, *Anagasta kuehniella* (Zeller), dark mealworm, *Tenebrio obscurus* (F.), and Cadelle beetle larva, *Tenebroides mauritanicus* (L.)] were obtained from cultures maintained at the U.S. Grain Marketing Research Laboratory where they are reared on whole or ground wheat media.

Hemolymph collection. Insects were anesthetized by cooling, and hemolymph was collected with a microcapillary pipet by cutting off and bleeding through a thoracic leg or the abdominal horn. A trace of 1-phenyl 2-thiourea was added to prevent clot formation. After the plasma was centrifuged at 10000*g* for 10 min at 4°C, it was stored frozen or immediately subjected to column chromatography.

Column chromatography and saccharide quantitation. Serum (0.1–0.5 ml) was gel filtered at 22°C on a column (0.9 or 1.5 cm × 120 cm) of Bio-Gel P-2[2] (Bio-Rad Laboratories, La Jolla, Calif.), equilibrated with 20 mM Tris (hydroxymethyl) aminomethane, 0.1 M NaCl, and adjusted to pH 7.3 by the addition of HCl. One-milliliter fractions were collected. Saccharide identity and purity were confirmed by thin-layer chromatography (1) on silica gel (Eastman) by using a solvent consisting of butanol, acetic acid, ethyl acetate, and water [9:6:3:1 (v/v)]. Sugars were quantitated by the anthrone reaction (13). Color values were compared with those obtained using trehalose (Sigma) and glucose (Sigma) as standards.

RESULTS AND DISCUSSION

In order to separate trehalose from glycoproteins and other saccharides present in insect blood, we utilized gel filtration on a polyacryl-

[2] Mention of a proprietary product does not constitute an endorsement by the U. S. Department of Agriculture.

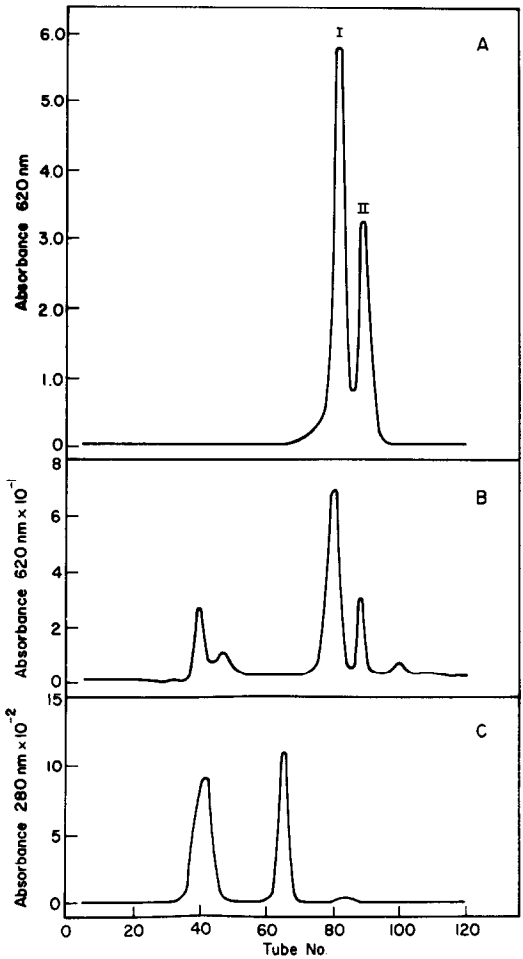

FIG. 1. Anthrone colorimetric analysis of eluate from Bio-Gel P-2 chromatography: (A) sugar mixture (Peak I, trehalose; Peak II, glucose); (B) 0.1 ml of *M. sexta* larvae hemolymph; (C) 280-nm absorbance profile of hemolymph components.

amide matrix. Figure 1A shows the result of a column standardization experiment in which a sugar mixture was separated on Bio-Gel P-2 and monitored by using the colorimetric anthrone condensation method (13). Trehalose (Peak I) and glucose (Peak II) were well resolved, with elution volumes of 80 ml (K_D = 0.68) and 87 ml (K_D = 0.79), respectively. Figure 1B depicts the elution profile obtained by chromatography of 0.1 ml of hemolymph from the fifth larval instar of the tobacco hornworm, *M. sexta*. Thin-layer chromatographic analysis revealed that Peak I was composed of trehalose only and that Peak II consisted of several monosaccharides, primarily glucose. The additional anthrone-positive

peaks in B are glycoprotein and glycopeptide components that eluted in or just after the void volume (38 ml) of the column. Figure 1C shows the ultraviolet absorbancy profile of hemolymph components (protein, peptide, and aromatic metabolites) for comparison. The limit of detectability of the Bio-Gel–anthrone (BGA) method was 2.5 μg of trehalose, or 0.1 ml of a 100 μM solution. The column chromatographic step was rate limiting in this procedure, and each sample required about 5 hr for processing. Routinely we utilized several columns to increase the sample capability of the method.

Wyatt (18) reported that the concentration of trehalose in insect hemolymph usually ranges from 20 to 180 mM, depending upon the species, developmental stage, and sex. We also surveyed several orders of insects for trehalose titer using the BGA method (Table 1) and found a wide range in concentration: moth larvae, 23–133 mM; beetle larvae, <0.1 to 8 mM; hemipteran nymph, 6 mM; and cockroach, 5–7 mM. The highest levels were found in lepidopteran insects in which the disaccharide was present at concentrations 5 to 10 times greater than those of several kinds of monosaccharides. In all the other species, monosaccharides were the major components, being present at concentrations 3 to 10 times higher than that of trehalose. The disaccharide titer of *P. americana* from our colony was approximately three times lower than

TABLE 1

TREHALOSE LEVELS IN INSECT HEMOLYMPH

Order	Insect	Stage[a]	Trehalose concentration (mM)[b]
Lepidoptera	*M. sexta*	Larvae	46 ± 5 (56)
		Pupae	23 ± 6 (4)
	M. quinquemaculata	Larvae	25 ± 4 (2)
	S. chersis	Larvae	133 ± 9 (2)
	P. interpunctella	Larvae	71 ± 7 (3)
	P. transitella	Larvae	62 ± 14 (2)
	C. cautella	Larvae	82 ± 15 (2)
	A. kuehniella	Larvae	87 ± 11 (2)
Coleoptera	*T. obscurus*	Larvae	8 ± 2 (2)
	T. mauritanicus	Larvae	<0.1 (5)
Hemiptera	*O. fasciatus*	Nymph	6 ± 2 (2)
Dictyoptera	*P. americana*	Adult	7 ± 2 (2)
		Nymph	5 ± 2 (2)

[a] Last larval or nymphal instar.

[b] Means ± SEM; number of determinations given in parentheses.

the values reported previously (2,8). The saccharide levels observed probably reflect the variable nutritional and developmental states of each species.

By using only a few microliters of hemolymph, this procedure can be utilized for direct trehalose titer determinations for most insects. It should be applicable to other fluids and tissues as well. We have successfully used the BGA method to assay for hypoglycemic factors in the neuroendocrine system of insects (11,17). However, in some other applications it may be necessary to stabilize trehalose by inhibiting any soluble trehalase in the hemolymph or tissue extracts (9,16). For this purpose several enzymatic inhibitors might be used, such as heavy metal ions or organic compounds, depending on the properties of the particular trehalase present (14,15). Nonenzymatic components that may interfere include monosaccharides, disaccharides, and nucleotides. These compounds should be resolved from trehalose in the gel-filtration step or be present at very low concentrations.

REFERENCES

1. Bassal, T. T. M. (1973) *J. Med. Entomol.* **10,** 228–9.
2. Bedford, J. J. (1977) *Comp. Biochem. Physiol.* **57A,** 83–86.
3. Bell, R. A. and Joachim, F. G. (1976) *Ann. Entomol. Soc. Amer.* **69,** 373–75.
4. Chippendale, G. M. (1978) *in* Biochemistry of Insects (M. Rockstein, ed.), Academic Press, New York, in press.
5. Dahlman, D. L. (1973) *Ann. Entomol. Soc. Amer.* **66,** 1023–1029.
6. Dahlman, D. L. (1975) *Comp. Biochem. Physiol.* **50A,** 165–167.
7. Fischl, J., Ishay, J., and Talmor, N. (1976) *Insect Biochem.* **6,** 53–58.
8. Florkin, M. (1937) *Bull. Soc. Chem. Biol.* **19,** 990.
9. Friedman, S. (1975) *Insect Biochem.* **5,** 151–164.
10. Giebel, P. E., and Domnas, A. J. (1976) *Insect Biochem.* **6,** 303–311.
11. Kramer, K. J., Tager, H. S., Childs, C. N., and Speirs, R. D. (1977) *J. Insect Physiol.* **23,** 293–295.
12. Matthews, J. R., Downer, R. G. H., and Morrison, P. E. (1976) *Comp. Biochem. Physiol.* **53A,** 165–168.
13. Roe, J. H. (1955) *J. Biol. Chem.* **212:** 335–343.
14. Sasajima, K., Kawachi, T., Sato, S., and Sugimura, T. (1975) *Biochim. Biophys. Acta.* **403,** 139–146.
15. Shimada, S. (1975) *Canad. Entomol.* **107,** 1311–1314.
16. Shimada, S. (1976) *Comp. Biochem. Physiol.* **54B,** 47–50.
17. Tager, H. S., Markese, J., Kramer, K. J., Speirs, R. D., and Childs, C. N. (1976) *Biochem. J.* **156:** 515–520.
18. Wyatt, G. R. (1967) *Adv. Insect Physiol.* **4,** 287–360.